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1-Butyl-3-methylimidazolium hydrogen sulfate catalyzed in-situ transesterification of *Nannochloropsis* to fatty acid methyl esters

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ABSTRACT

1-Butyl-3-methylimidazolium hydrogen sulfate ([Bmim][HSO_4]) is used as a solvent and an acid catalyst for in-situ extractive transesterification of wet Nannochloropsis with methanol. The reaction is supposed to be a five-step process: (1) wet algae cell wall dissolves in ionic liquid at reaction temperatures; (2) hydrogen ions and sulfate ions release from the ionization of HSO_4. The hydrogen ions (H^+) act as catalysts for accelerating the reactive extraction of triglyceride from wet Nannochloropsis; (3) hydrogen ions and methanol molecules transfer from bulk to active site of cells without passing through cell wall that is dissolved by ionic liquid; (4) in-situ transesterification of lipid (mainly triglycerides) with methanol; and (5) products transfer from inside of algae cells to outside of cells. The crude biodiesel yield of [Bmim][HSO_4] catalyzed in-situ transesterification is about 95.28% at reaction temperature of 200 °C, reaction time of 30 min, mass ratio of [Bmim][HSO_4] to wet Nannochloropsis of 0.9:1, and a mass ratio of methanol to wet algae of 3:1. It decreases to 81.23% after [Bmim][HSO_4] is recycled for 4 times, which indicates that [Bmim][HSO_4] catalyzed in-situ transesterification is an economic approach for biodiesel production from wet algae.

1. Introduction

The expansion of world population has driven a critical appetite of human societies for energy [1]. The transport sector energy demand is expected to increase 60% by 2030 due to the sector expanding in the USA, Europe, and some other new industrialized and emerging economics of China and India [2]. Biofuel is a promising renewable energy source to reduce fossil fuel consumption and greenhouse gas emissions [3]. However, the competition of biofuel with food production leads to series of problems, such as fluctuation of food price, overuse of land, high cost in energy and carbon emissions [4]. Algae as new alternative energy sources with low land use have attracted much attention for more than 50 years [5]. Compared with other kinds of energy crops, algae are more promising alternative feedstocks for biodiesel production due to their following properties: (1) Algae have rapid growth rate. They are reported to be the fastest-growing plants in the world [6]. The doubling time of microalgae range between 3.5 and 24 h during exponential growth phase [7]. (2) Algae have high annual product. Algae can be cultured all the year round with high quantity of oil production of 58,700 l ha^{-1}, which are much higher than the best oilseed crops [8]. (3) Algae are environmental friendly biofuel feedstock. They can be cultured in brackish aqueous on non-arable land, avoiding land use competition with terrestrial crops [9]. And also, algae cultivation can minimize associated environmental impact caused by fossil fuel combustion. (4) Algae have high oil content. The oil content is about 20–90% of dry weight of algae depending on species and growth conditions [10]. (5) Algal biofuel have high value added co-products. The lipid extracted algae (LEA) can be as a source of protein and reduced sugar [11], and maltodextrin products [12]. Also, algae have commercial applications as natural sources of carotenoids, phycocolloids, etc. [13]. Algae may also assist to solve four main challenges facing in modern society: security of energy, water, food supplies and climate protection [14].

Bioenergy production routes can be classified as biochemical, chemical and thermo-chemical processes [15]. Comparing with other technologies, such as microwave-assisted pyrolysis [16],

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and solid-state fermentation [17], transesterification reaction of lipid extracted from algae with alcohols is currently the most accessible technology for biodiesel production [18]. Algal biodiesel is usually generated through acidic or base catalyzed transesterification after pretreatment and extraction [19]. However, the extensive upstream and downstream processes, such as dehydration from algae, extraction, and purity processes, make biodiesel production from algae an expensive process [20]. In-situ reactive extraction process is proposed to be a preferred alternative to directly process the alcoholysis of the oil in algae avoiding the extraction step to produce biodiesel from algae in conventional method [21], as well as safety concerns in supercritical technology [22]. The effect of ionic liquids as excellent solvents on lipid extraction from algae has been studied in few previous works. Ionic liquids mixtures with different anions were favorable to lipid extraction from Chlorella [23]. In another work, 1-ethyl-3-methylimidazolium methylphosphate was found to be the best solvent of marine microalgae without heating [24]. 1-Butyl-3-methylimidazolium methylsulfate assisted lipid extraction from Chlorella vulgaris was also proved to be 1.6 times higher than ultrasound irradiation assisted extraction [25]. It was reported by Lee that [Bmim][HSO_4] and [Bmim][CF_3SO_3] were more effective in algal biodiesel production than other ionic liquids due to their low lipid solubility [26]. Also, acid ionic liquids can be used as acid catalysts for transesterification avoiding soap formation and emulsion caused by alkali catalysts usage [27]. However, few studies have been done to investigate the effect of acid ionic liquids on in-situ transesterification process.

In this work, 1-butyl-3-methylimidazolium hydrogen sulfate ([Bmim][HSO_4]) is applied both as a solvent for lipid extraction and an acid catalyst for in-situ transesterification of triglyceride in Nannochloropsis along with methanol. The effects of reaction temperature, reaction time, and mass ratio (wet algae: 1-butyl-3-methylimidazolium hydrogen sulfate) on biodiesel yield are investigated thoroughly. The compositions of crude biodiesel products have been studied by a gas chromatograph (GC) connected with an Agilent 5975 series mass selective detector (MSD) system. To further assess effects of different processing procedures on wet Nannochloropsis biomass, microscopic imaging with fluorescent probes is used to visualize the integrity of algal cells, extent of cell walls, and hydrophobic compartments in the residual algal cells.

2. Materials and methods

2.1. Materials

Nannochloropsis sp. was obtained from Solix biofuels (Fort Collins, CO) and stored at −5 °C before use. The freezing wet algae sludge clusters with moisture content of 62% were used directly without any pretreatment. The algae cell diameter was about 2–3 μm by transmission electron microscope (TEM) analysis. The total lipids content was determined to be 52% on the dry weight basis by the Folch method [28]. Methanol (99.9%, v/v), purchased from Sigma-Aldrich, was used for in-situ transesterification directly. The in-situ transesterifications of Nannochloropsis with methanol were carried out in the PARR 4593 Micro-reactor with a 4843-controller (Parr Instrument Company, Illinois, USA). The SPE silica columns, purchased from Thermo Scientific (Waltham, MA), was used to remove pigments from crude FAME biodiesel product. After removal of unreacted alcohols in the products by evaporation in a vacuum oven, thermo-gravimetric analysis (TGA) of FAME biodiesels obtained from in-situ transesterification of Nannochloropsis was performed using Perkin Elmer Pyris 1 TGA. Heptane (⩾99.5%, GC) was used as a solvent for GC-MS analysis. And the composition of crude biodiesel obtained was characterized by GC-MS.

2.2. In-situ transesterification of wet Nannochloropsis with methanol

The in-situ transesterification of wet Nannochloropsis with methanol was conducted in a 100 mL PARR reactor. Algae samples were directly mixed with methanol and [Bmim][HSO_4] at room temperature for in-situ transesterification without any pretreatment. The reactor was then pressurized to 200 psi (1.38 MPa) by injecting compressed N_2 after addition of reactants in all experiments. The parameters, reaction temperature of 100–200 °C, reaction time of 0–180 min, and mass ratio of algae with [Bmim][HSO_4] of 0.3, 0.6, 0.9–1.2, were varied to study their effects on crude biodiesel yield. After transesterification process, *n*-hexane was used to separate crude biodiesel products with residual and aqueous phase. The crude biodiesel was then purified using a silica column to remove pigments for following GC-MS analysis. The aqueous phase containing residue was centrifuged to separate algal residual with aqueous phase, and the residual was then dried in an oven at 80 °C for TEM imaging analysis.

2.3. Physical properties analysis of biodiesel product

The thermo-gravimetric analyses (TGA) of crude FAME biodiesel and [Bmim][HSO_4] were performed using Perkin Elmer Pyris 1 TGA. The samples were heated from 30 °C to 800 °C at a constant heating rate of 10 °C/min in an atmosphere of nitrogen and a constant purge rate of 20 mL/min at the pan.

The calorific value of crude biodiesel was tested using a Parr 6725 semi-micro calorimeter. The bomb was filled with oxygen to 35 bar (3500 kPa), and then the bomb was put into a tank filled with 959.42 g of water. The operation mode was then chosen to test the calorific value of biodiesel combusted in bomb.

2.4. Preparation of transmission electron microscopy (TEM) samples

The untreated suspensions of Nannochloropsis cells and lipid extracted algae (LEA) under reaction temperature of 175 °C, reaction time of 30 min, and a mass ratio of methanol to wet Nannochloropsis of 3:1 conditions after frozen storage and processing for embedding were characterized by TEM. Algal biomass powder was hydrated in 0.1 M imidazole HCl buffer, then fixed and embedded for thin sections according to an established procedure for preserving lipids. The TEM images showed profiles of algal cells containing large, electron-dense oil droplets representing large lipid droplets as well as other cellular organelles.

2.5. Confocal images of calcofluor white and Nile red stained algae samples

Microscopic localization of calcofluor white (CW) fluorescence (Sigma-Aldrich) was used to evaluate the integrity of wet Nannochloropsis cell walls after lipid extraction. Approximately, 0.8 mL of algae biomass with 0.2 mL of CW stain was suspended into 0.1 M imidazole buffer at pH = 7.2, and incubated in the dark for 15 min before 10 μL samples were transferred to the coverslip of glass bottom microwell dishes for confocal imaging using a model TCS SP5 confocal microscope system (Leica Microsystms, Exton, PA). Matching images of fluorescence from algal biomass fractions were collected sequentially: calcofluor white fluorescence was excited at 405 nm and emission was collected from 415 to 475 nm in section volumes ranging from 20 to 30 μm. For comparison, images of cellular autofluorescence from excitation at 488 nm were collected simultaneously in two channels from 510 to 570 nm and 660 to 720 nm.

The lipid droplets of wet Nannochloropsis and residue after lipid extraction were measured using Nile red fluorescence. Approximately, 100 μL of algal cell suspensions were suspended in 2.5% glutaraldehyde–0.1 M imidazole HCl buffered solution at pH = 7.2. Small aliquots (2–5 μL) of the suspended algal cells were deposited in multiwell dishes (MatTek Corp., Ashland, MA), and images of fluorescence were collected with a laser scanning confocal microscope using the 488 nm line from an argon laser for excitation and two channels of emission (500–550 nm for non-specific autofluorescence and 670–690 nm for chlorophyll autofluorescence) and a 63× oil immersion lens. Spectra of autofluorescence from 500 to 750 nm was measured before and after the addition of 100 μg mL^{-1} Nile Red to an aliquot of the algal cell suspension, and images with three emission channels were obtained and shown in Fig. 2 of supporting information, 500–550 nm, 580–620 nm (Nile Red fluorescence) and 670–690 nm. The addition of Nile Red to algal suspension highlights large areas of yellow fluorescence in many cells, which probably represented lipid droplets based on the known lipophilic staining of Nile Red.

2.6. GC-MS analysis

For the quantification of reaction products, the crude biodiesel samples obtained by in-situ transesterification were analyzed by an Agilent 7890A gas chromatograph (GC) connected to an Agilent 5975 series mass selective detector (MSD) system with a capillary column (DB-23, 60 m × 250 μm × 0.15 μm nominal). The contents of biodiesels were calculated quantitatively by an internal standard method. Methyl tricosanoate (C23:0.99%), purchase from Sigma Aldrich (St. Louis, MO), is used as internal standard for quantification of the compositions of fatty acid methyl esters. Approximately, 1 μL sample was injected into the gas chromatograph with constant flow rate (1 mL/min) helium as the carrier gas. The injection was performed in split mode (50:1). The parameters of the oven temperature program consisted of: starting at 50 °C with 10 °C/min intervals up to 220 °C (1 min) and up to 250 °C with 5 °C/min intervals (2 min). The temperature of the injector and detector were set at 250 °C. The identified components of the fatty acid methyl esters were listed in Table 1. The fatty acid esters content was calculated by using the following equation: $C = \frac{(\sum A) - A_{EI}}{A_{EI}} \times \frac{C_{EI} \times V_{EI}}{W} \times 100\%$, where $\sum A$ represents total peak area of fatty acid esters, A_{EI} represents the peak area of internal standard (C23:0), C_{EI} (mg/mL) is the concentration of internal standard, V_{EI} is the volume of the solution of internal standard, and W is the total weight of the sample [29]. The crude biodiesel yields and FAME percentage in crude extract were calculated using the formulae given below:

$$\text{Crude biodiesel yield} = \frac{\text{weight of crude extract}}{\text{weight of lipid in algae samples}} * 100$$

$$\text{FAME percentage in crude extract} = \frac{\text{Weight of FAMEs}}{\text{Weight of crude biodiesel}} * 100$$

Table 1
Identified compounds of crude FAME biodiesel by GC-MS analysis.

Fatty acid esters	Common name	Retention time (min)
Octanoic acid	C8:0	6.542
Decanoic acid	C10:0	8.576
Methyl tetradecanoate	C14:0	9.776
Pentadecanoic acid, methyl ester	C15:0	10.476
Hexadecanoic acid, methyl ester	C16:0	11.327
9-Hexadecenoic acid, methyl ester, (*Z*)-	C16:1	11.601
7,10-Hexadecadienoic acid, methyl ester	C16:2	11.921
Heptadecanoic acid, methyl ester	C17:0	12.207
Methyl 7,10,13-hexadecatrienoate	C16:3	12.498
Octadecanoic acid, methyl ester	C18:0	13.227
9-Octadecenoic acid, methyl ester, (*E*)-	C18:1	13.507
9,12-Octadecadienoic acid (*Z,Z*)-, methyl ester	C18:2	14.049
9,12,15-Octadecatrienoic acid, methyl ester, (*Z,Z,Z*)-	C18:3	14.754
5,8,11,14-Eicosatetraenoic acid, methyl ester, (all-*Z*)-	C20:4	17.103
cis-5,8,11,14,17-Eicosapentaenoic acid, methyl ester	C20:5	17.966
Tricosanoic acid, methyl ester	C23:0	19.44

3. Results and discussions

3.1. Thermo-gravimetric and calorific value analysis of biodiesel products

As was shown in Fig. 1 of supporting information, there was one weight loss phase of [Bmim][HSO_4] thermo-gravimetric analysis curve in the temperature range of 250–300 °C, which represented that [Bmim][HSO_4] was thermally stable up to 250 °C. Therefore, [Bmim][HSO_4] can be recycled efficiently for in-situ transesterification of wet algae with methanol. There were two phases of weight loss in crude biodiesel yield: the first phase was in temperature range of 150–200 °C; the second phase was in the range of 300–400 °C. This was due to different vapor pressures and thermal stability of saturated and unsaturated fatty acid esters. The unsaturated fatty acid esters were easier to decompose compared with saturated fatty acid esters. And thus, there were two weight loss phases presented in thermal-gravimetric analysis curves.

The calorific value of crude biodiesel was tested in this work. The calorific value of biodiesel was 39.11 MJ/kg, indicating that FAME products of in-situ transesterificated algae products were high value added biofuel in practical use.

3.2. TEM analysis of wet Nannochloropsis and lipid extracted algae

The TEM analysis of raw wet Nannochloropsis and LEA under reaction temperature of 175 °C, reaction time of 30 min, a mass ratio of methanol to wet Nannochloropsis of 3:1 conditions, with and without [Bmim][HSO_4] as co-solvent, were shown in Fig. 1 (a)–(c). Transmission electron microscopy of untreated suspensions of Nannochloropsis cells after frozen storage and processing for embedding reveals well-separated, irregular circles and ovoid profiles of intact cells bounded by a clear cell wall with diameters measuring from 2 to 3 μm (Fig. 1(a)), and profiles contain large inclusions that identify as lipid based on stained electron density [30]. After subcritical methanol extraction of wet algae under temperature of 175 °C, reaction time of 30 min, and a mass ratio of methanol to wet Nannochloropsis of 3:1, separated profiles of Nannochloropsis cells were found with reduced diameters ranging from 1 to 2 μm, cell contents were homogeneous with a uniform electron density and contained within an intact through convoluted profile of the cell wall (Fig. 1(b)). In Fig. 1(c), thin sections of cellular suspensions treated for 30 min in [Bmim][HSO_4], were composed of large millimeter-sized clumps of cellular materials containing particles with two different segregated distributions. One type of material consisted of distinct loosely packed particles around 1 μm in diameter with a uniform electron density, and the other consisted of densely packed irregular particles also around 1 μm in diameter; these often surrounded or separated by convoluted profiles of membranous-like sheets and occasionally particles resembling distorted chloroplasts. The wet algae cell wall was dissolved in ionic liquid according to TEM characterization.

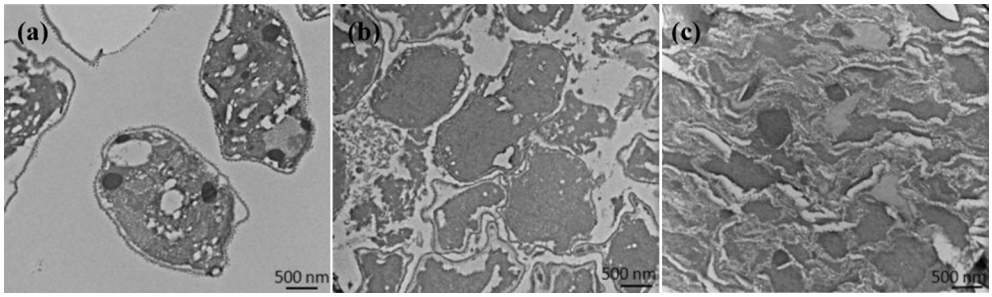

Fig. 1. TEM images of wet Nannochloropsis and the lipid extracted algae with the temperature of 175 °C, the reaction time of 30 min, and the mass ratio of methanol to wet Nannochloropsis of 3:1. (a) Raw wet Nannochloropsis sp.; (b) lipid extracted algae without [Bmim][HSO_4] as solvent; and (c) [Bmim][HSO_4] - assisted lipid extracted algae with the mass ratio of [Bmim][HSO_4] to wet Nannochloropsis of 0.9:1.

At the same time, [Bmim][HSO_4] ionized to form hydrogen ions in wet algae sludge. Hydrogen ions and methanol molecules transfer from bulk to active site of cells without passing through cell wall. The dissolved cell wall was favored to the mass transfer of hydrogen ions and methanol molecules from bulk to active site of algae cells. These were insistent with first three steps of proposed mechanism in abstract. The comparison of TEM images of raw wet Nannochloropsis, non-ILs assistant extracted algae, and ILs-assistant LEA directly indicated that [Bmim][HSO_4] was an excellent solvent of cellulose and hemicellulose, which were major components of cell walls.

3.3. Calcofluor white fluorescence tests of Nannochloropsis and LEA

Microscopic localization of calcofluor white (CW) fluorescence (Sigma-Aldrich) was used to evaluate the integrity of wet Nannochloropsis cell walls after lipid extraction. Confocal images of raw wet Nannochloropsis with calcofluor white (Fig. 2(a)), non-[Bmim][HSO_4] assisted LEA with calcofluor white (Fig. 2(b)), and [Bmim][HSO_4] assisted LEA with calcofluor white (Fig. 2(c)), were illustrated in Fig. 2. Images of cellular autofluorescence from excitation at 488 nm were collected simultaneously in three channels from 415 to 475 nm, 510 to 570 nm and 660 to 720 nm to study the stain of algae cells in detail. As was shown in Fig. 2(a), a few whole cells with calcofluor white fluorescence were marked by intense fluorescence, however, most of the surrounding cells were clearly labeled with CW at much lower levels possibly due to the ages of different cells in the sample population. Comparing with Fig. 2(a), more algae cells were intensely labeled in Fig. 2(b) after non-[Bmim][HSO_4] assisted in-situ reaction due to the destruction of cell walls. Intact cell walls and cells were still observed after in-situ reaction without [Bmim][HSO_4] as a co-solvent and catalyst (Fig. 2(b)). However, as illustrated in Fig. 2(c), cell walls were totally destroyed in [Bmim][HSO_4] assisted reaction, and no whole cells were observed after in-situ transesterification. Only some areas of biomass were stained with calcofluor white. The comparison of images of calcofluor white fluorescence from wet algae, non-[Bmim][HSO_4] assisted LEA, and [Bmim][HSO_4] assisted LEA demonstrated that [Bmim][HSO_4] was an effective co-solvent of the cell wall. It also illustrated that cell wall was destroyed in [Bmim][HSO_4] assisted in-situ reaction, which was helpful to increase reaction rate of in-situ transesterification.

3.4. Effect of in-situ transesterification temperature

The effect of reaction temperature on crude biodiesel yields of [Bmim][HSO_4] catalyzed and non-[Bmim][HSO_4] catalyzed in-situ transesterification process was shown in Fig. 3(a). All experiments were conducted at constant mass ratio of [Bmim][HSO_4] to wet algae of 0.3 and reaction time of 30 min, while the reaction temperatures varying from 100 °C to 200 °C, with 25 °C as interval value. The reactants were heated up to subcritical conditions with average heat rates of 6 °C/min, and were stirred at a constant stir rates of 350 r/min. As presented in Fig. 3(a), there was a significant increase of crude biodiesel yield with increase of reaction temperature in both [Bmim][HSO_4] catalyzed in-situ transesterification and non-catalyzed transesterification. Another point worth mention was that crude biodiesel yields of [Bmim][HSO_4] catalyzed in-situ transesterification was much higher than that of non-[Bmim][HSO_4] assisted subcritical methanol reaction in temperature range of 100–200 °C, which indicated that [Bmim][HSO_4] helped to shift the equilibrium of transesterification to the production of biodiesel. There were three reasons to explain the higher crude biodiesel yield of [Bmim][HSO_4] catalyzed in-situ transesterification than that of non-[Bmim][HSO_4] catalyzed in-situ transesterification. Firstly, the temperature was favorable to crude biodiesel yield because transesterification was an endothermic process. Secondly, [Bmim][HSO_4] was thermal stable in the range of 100–200 °C observed through a thermo-gravimetric analysis of [Bmim][HSO_4]. Thirdly, [Bmim][HSO_4] dissolved algae cell walls through TEM image analysis, which assisted in-situ transesterification of Nannochloropsis.

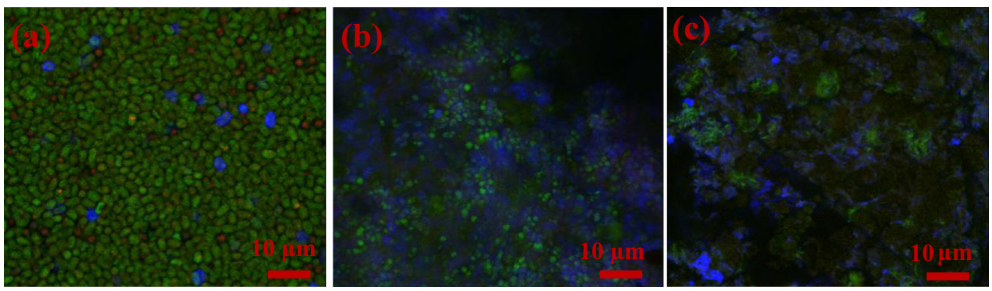

Fig. 2. Confocal microscopy images of Nannochloropsis with calcofluor white. (a) Raw wet Nannochloropsis; (b) non-[Bmim][HSO_4] assisted in-situ transesterification residuals; and (c) [Bmim][HSO_4] assisted in-situ transesterification residuals.

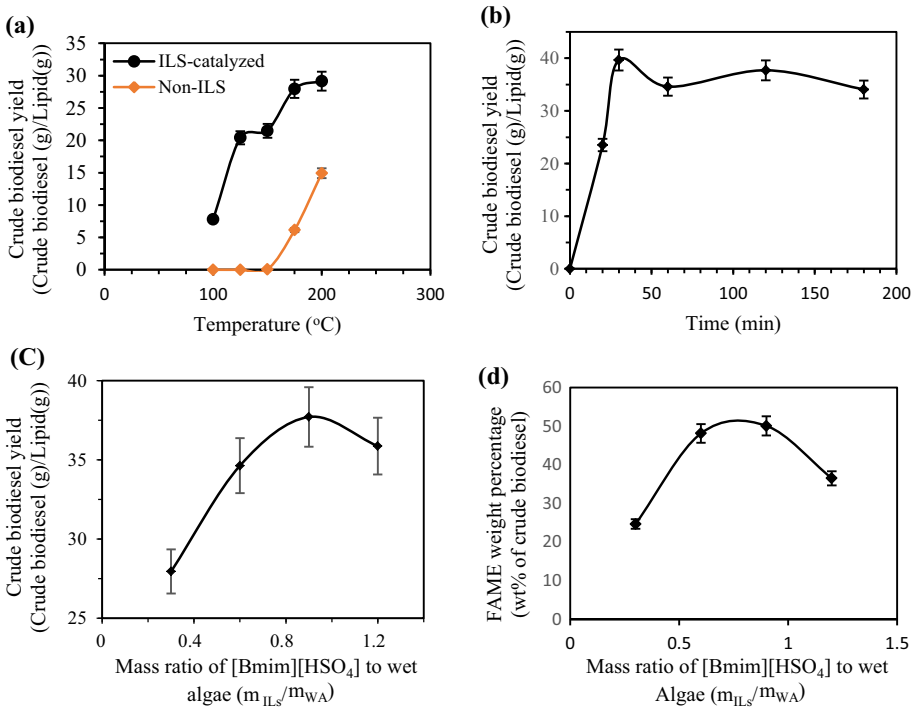

Fig. 3. (a) Influence of reaction temperature on yield of FAME; (b) influence of reaction time on crude biodiesel yield; (c) influence of mass ratio on crude FAME yield; and (d) the change of FAME weight percentage with the change of mass ratio of [Bmim][HSO_4] to wet Nannochloropsis.

Nile red fluorescence was used to illustrate the distribution of lipid in algae cells and LEA. Confocal images of raw wet Nannochloropsis without Nile red fluorescence (Fig. 4(a)) and with Nile red fluorescence (Fig. 4(b)), and Nile red fluorescence stained non-[Bmim][HSO_4] assisted LEA (Fig. 4(c)) and [Bmim][HSO_4] assisted LEA (Fig. 4(d)), were shown in Fig. 4. The wet Nannochloropsis stained by Nile red shown in Fig. 4(b) illustrated the distribution of lipid in algae cells. After non-[Bmim][HSO_4] assisted in-situ reaction, the lipid extracted algal also fluoresced with Nile red, however, the fluorescence was diffuse and much less intense than that of the raw algae with Nile red. The same phenomena were also observed in confocal images of [Bmim][HSO_4] assisted LEA (Fig. 4 (d)) comparing with that of raw wet algae. Gray level histograms using Fovea Pro v4.0 image analysis and processing software (Reindeer Graphics Inc., Ashville, NC) of images in Fig. 4(b) –(d) were plotted and compared in Fig. 3 of supporting information. As was shown in Fig. 3 of supporting information, the gray level of images was decreasing from Fig. 4(b)–(d). It indicated that the relative lipid droplets in LEA content was markedly decreased from raw wet algae (Fig. 4(b)) to [Bmim][HSO_4] assisted LEA (Fig. 4(d)). This proved that [Bmim][HSO_4] was both an effective co-solvent of cell wall and an efficient catalyst of in-situ reaction.

3.5. Effect of in-situ transesterification time

The reaction time was also a significant factor to affect the reversible in-situ transesterification process. All experiments were carried out at constant reaction temperature of 175 °C, mass ratio of methanol to wet algae of 3:1. The mass ratio of [Bmim][HSO_4] to wet algae was held at 0.9 since a maximum crude biodiesel yield was obtained at constant mass ratio of [Bmim][HSO_4] to wet algae of 0.9. The reaction time varied from 0 min to 20 min, 30 min, 60 min, 120 min, and 180 min for the study of effect of reaction time on crude biodiesel yield. Fig. 3(b) showed the change of crude biodiesel yield with extension of reaction time. The crude biodiesel yield was about 24 wt% at reaction time of 20 min. The crude biodiesel yield increased up to 39 wt% at reaction temperature of 30 min, and the crude biodiesel yield maintained almost the same after reaction time was longer than 30 min, which indicated that [Bmim][HSO_4] was an effective catalyst of in-situ transesterification of wet Nannochloropsis. The mechanism of [Bmim][HSO_4] catalyzed in-situ transesterification of wet Nannochloropsis was shown in Fig. 5, which was much similar with mechanism of homogeneous acid catalyzed in-situ reaction presented in the previous work [31]. [Bmim][HSO_4] catalyzed transesterification of wet Nannochloropsis with methanol had the following five steps: the ionization of [Bmim][HSO_4]; the ionization of hydrogen sulfate; protonation of the carbonyl group of the triglyceride; nucleophilic attack of the alcohol; and the proton migration and breakdown of the intermediate. These five steps shown in Fig. 5 were repeated for three times to step wisely convert triglyceride to diglyceride, monoglyceride, and glycerin [31].

3.6. Effect of mass ratio of [Bmim][HSO_4] to wet Nannochloropsis

The effect of mass ratio of [Bmim][HSO_4] to wet Nannochloropsis on crude biodiesel yield was shown in Fig. 3(c). The reaction temperature maintained at 175 °C with reaction time of 30 min, while the mass ratio of [Bmim][HSO_4] to algae was varied from 0.3, 0.6, 0.9 to 1.2 to study effect of mass ratio on crude biodiesel yield. As can be seen in Fig. 3(c), the crude biodiesel yield was increased from 28 wt% to 37 wt% when mass ratio of [Bmim][HSO_4] to wet algae increase from 0.3 to 0.9. The increase of crude biodiesel yields with the increase of mass ratio of [Bmim][HSO_4] to wet algae indicated that [Bmim][HSO_4] favored to the crude biodiesel production. However, the crude biodiesel yield was only about 37 wt% due to low reaction temperature, short reaction time, and moisture content of wet algae. However, there was a slight decrease of crude biodiesel yield after the maximum yield was obtained at a mass ratio of [Bmim][HSO_4] to wet algae of 0.9. The change of FAME weight percentage of crude biodiesel with the variation of mass ratio of [Bmim][HSO_4] to algae was also studied

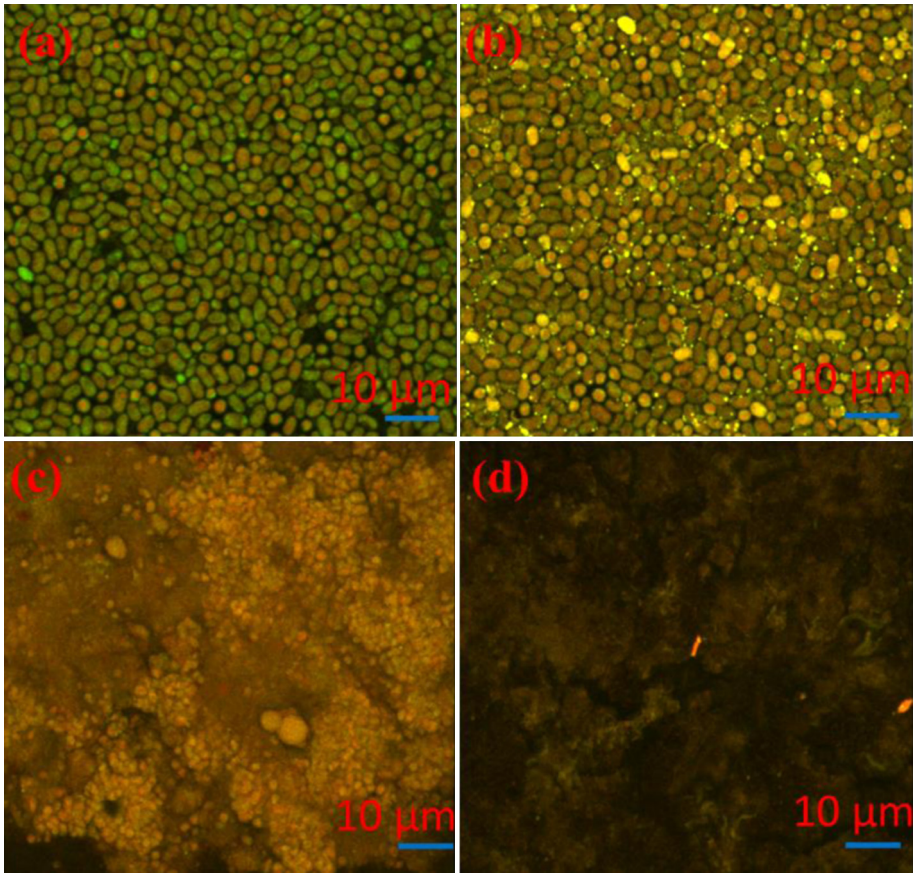

Fig. 4. Confocal microscopy images of: (a) wet Nannochloropsis without Nile red; (b) wet Nannochloropsis with Nile red; (c) non-[Bmim][HSO_4] assisted lipid extracted algae with Nile red; and (d) [Bmim][HSO_4] assistant lipid extracted algae with Nile red.

and presented in Fig. 3(d). The same trend was also observed with the variation of crude biodiesel yield with different mass ratio of [Bmim][HSO_4] to algae. The increase of mass ratio of [Bmim][HSO_4] to algae was favorable to FAME production initially, while there was a slightly decrease of the FAME yield after the mass ratio of catalyst to wet algae increased to 0.9. Therefore, the mass ratio of [Bmim][HSO_4] to wet algae of 0.9 was supposed to be the optimum reaction condition for [Bmim][HSO_4] catalyzed in-situ transesterification of wet Nannochloropsis. These results indicated that [Bmim][HSO_4] had a positive effect on biodiesel production due to the following two reasons: on the one hand, the [Bmim][HSO_4] was an excellent solvent of cellulose and hemicelluloses, and thus it would be an effective solvent to break the cell wall of wet algae. Therefore, the solubility of the cell wall of microalgae in [Bmim][HSO_4] may have facilitated the lipid extraction from wet algae; On the other hand, since the transesterification is a reversible reaction, the biodiesel production process can be catalyzed by acid or basic catalysts [32]. [Bmim][HSO_4], an acidic ionic liquid, was presumed to be an acid catalyst of the in-situ transesterification of wet Nannochloropsis with methanol through its higher yield than non-[Bmim][HSO_4] assisted reaction. Therefore, the increased amount of [Bmim][HSO_4] had a positive effect on crude biodiesel yield production and FAME percentage of crude biodiesel, initially. However, when mass ratio of [Bmim][HSO_4] to algae was over 0.9, it was supposed that extra [Bmim][HSO_4] would be absorbed out of the cells. Lipid cannot be dissolved in ionic liquid, while methanol molecules, soluble in [Bmim][HSO_4], would mixed together with [Bmim][HSO_4] out of algae cells. Thus, extra [Bmim][HSO_4] blocked the interaction of methanol with lipid in wet algae. And the FAME weight percentage in crude biodiesel products decreased a little after the maximum FAME weight percentage was obtained.

3.7. Recycling of [Bmim][HSO_4]

[Bmim][HSO_4] catalyzed in situ transesterification of wet Nannochloropsis with methanol after hydrogen ions and methanol molecules transfer from bulk to active site of cells. [Bmim][HSO_4] and crude biodiesel obtained were then separated into organic phase and aqueous phase after in situ reaction without any effect to algal residuals, separately. The ionic liquid in aqueous phase was collected after filtration of precipitation of algae. [Bmim][HSO_4] was then dried by vacuum oven at 60 °C for further test of its reusability. All recycling experiments were conducted for 30 min with mass ratio of methanol to wet algae of 3:1, and mass ratio of [Bmim][HSO_4] to wet Nannochloropsis of 0.9. The recycling test of [Bmim][HSO_4] was carried out at reaction temperature of 200 °C since it's easier to form a homogeneous mixture under supercritical and subcritical conditions. Also, the higher mass ratio of [Bmim][HSO_4] to wet Nannochloropsis helped to improve reaction efficiency through high hydrogen bond acidity of [Bmim][HSO_4] under high temperature [33]. As can be seen in Fig. 6, the crude biodiesel yields of original [Bmim][HSO_4] assisted in-situ transesterification was 95.28%, and it decreased to 92.79% when recycled [Bmim][HSO_4] was used to catalyze in-situ transesterification for the second time. The crude biodiesel yield step wisely decreased to 81.23% after it was reused for 4 times, which may be due to the decrease of the purity of the ionic liquid [23]. However, the crude biodiesel yield was still presented to be much

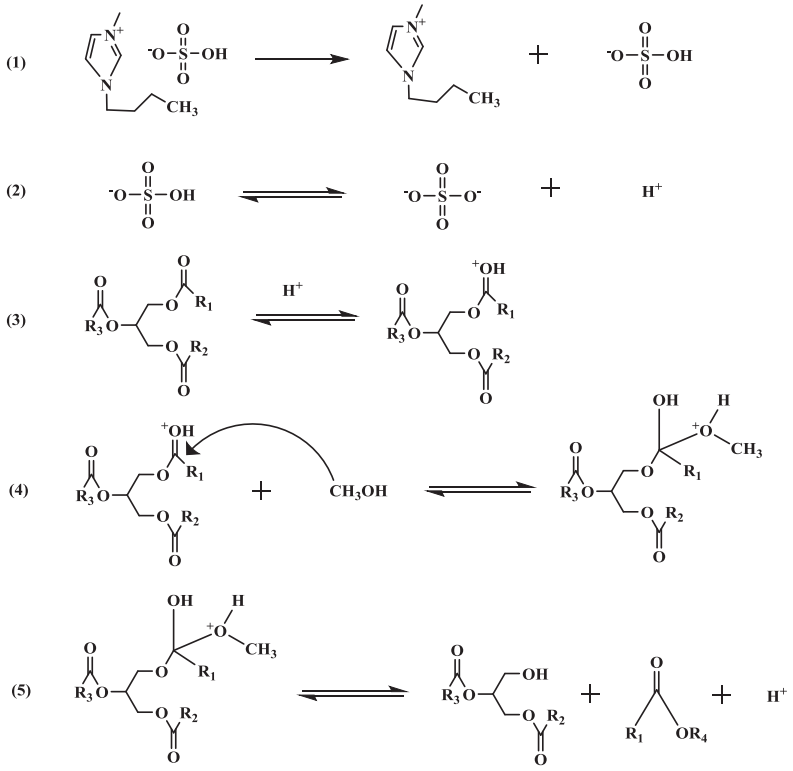

Fig. 5. Mechanism of the [Bmim][HSO_4] catalyzed in-situ transesterification of wet Nannochloropsis with methanol. (1) Ionization of [Bmim][HSO_4]; (2) ionization of HSO_4^-; (3) protonation of the carbonyl group of the triglyceride by the acid catalyst; (4) nucleophilic attack of the alcohol; and (5) proton migration and breakdown of the intermediate.

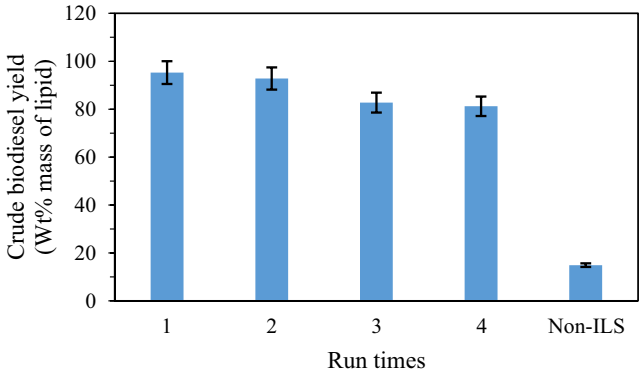

Fig. 6. Reusability of [Bmim][HSO_4] catalysts. The reaction time is 30 min, with reaction temperature of 200 °C, mass ratio of [Bmim][HSO_4] to wet algae of 0.9:1, and mass ratio of methanol to wet Nannchloropsis of 3:1. Non-ILs represents the in-situ transesterification without ILs as catalyst.

higher than that of non-ILs catalyzed reaction. The results indicated that [Bmim][HSO_4] was an efficient catalyst of in-situ transesterification of wet algae. Also, [Bmim][HSO_4] was thermal stable and can be recycled for the in-situ transesterification with high catalytic efficiency.

4. Conclusions

In this work, one-step [Bmim][HSO_4] catalyzed extractive reaction of wet Nannochloropsis with methanol has been studied in detail. The effects of reaction temperature, reaction time, and mass ratio of wet algae to 1-butyl-3-methylimidazolium hydrogen sulfate on crude biodiesel yield were investigated. The reaction temperature was favorable to the crude biodiesel yield in the temperature ramp of 100–200 °C. The results also proved that [Bmim][HSO_4] catalyzed in-situ transesterification can reach the optimum yield in a short time (30 min). The crude biodiesel yields also increased with an increase of the mass ratio of [Bmim][HSO_4] to wet algae initially; however, the crude biodiesel yield decreased a little after the mass ratio was over 0.9. [Bmim][HSO_4] was concluded both a solvent for lipid extraction and an effective acid catalyst for in-situ transesterification in this work. [Bmim][HSO_4] was also recycled for 4 times to test its reusability. The crude biodiesel yield was 81.23% after it was recycled for 4 times, which indicated that [Bmim][HSO_4] was thermal stable and effective for in-situ transesterification of wet Nannochloropsis with methanol.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enconman.2016.10.071.

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